Redox Biology 4 (2015) 74–86

Contents lists available at ScienceDirect

Redox Biology

journal homepage: www.elsevier.com/locate/redox

Research Paper

Functional interaction between cyclooxygenase-2 and p53 in response to an endogenous electrophile

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ARTICLE INFO

Article history:
Received 7 November 2014
Received in revised form
27 November 2014
Accepted 29 November 2014
Available online 6 December 2014

Keywords:
4-Hydroxy-2-nonenal
Cyclooxygenase
p53
Lipid peroxidation
Proteasome
Sp1

ABSTRACT

Cyclooxygenase-2 (Cox-2) is rapidly expressed by various stimuli and plays a key role in conversion of free arachidonic acid to prostaglandins. We have previously identified 4-hydroxy-2-nonenal (HNE), a lipid peroxidation-derived electrophile, as the potent Cox-2 inducer in rat epithelial RL34 cells and revealed that the HNE-induced Cox-2 expression resulted from the stabilization of Cox-2 mRNA that is mediated by the p38 mitogen-activated protein kinase signaling pathway. In the present study, we investigated an alternative regulatory mechanism of Cox-2 expression mediated by a transcription factor p53. In addition, to characterize the causal role for Cox-2, we examined the effects of Cox-2 overexpression in RL34 cells. To examine whether the HNE-induced Cox-2 expression was mechanistically linked to the p53 expression, we analyzed changes in Cox-2 and p53 expression levels in response to HNE and observed that the Cox-2 levels were inversely correlated with the p53 levels. Down-regulation of p53 followed by the activation of a transcription factor Sp1 was suggested to be involved in the HNE-induced Cox-2 gene expression. To characterize the effect of Cox-2 expression in the cells, we established the Cox-2-overexpressing derivatives of RL34 cells by stable transfection with Cox-2 cDNA. An oligonucleotide microarray analysis revealed a dramatic down-regulation of the proteasome subunit RC1 in the Cox-2 overexpressed cells compared to the empty-vector transfected control cells. Consistent with the Cox-2-mediated down-regulation of proteasome, a moderate reduction of the proteasome activities was observed. This proteasome dysfunction mediated by the Cox-2 overproduction was associated with the enhanced accumulation of p53 and ubiquitinated proteins, leading to the enhanced sensitivity toward electrophiles. These results suggest the existence of a causal link between Cox-2 and p53, which may represent a toxic mechanism of electrophilic lipid peroxidation products.

Introduction

Cyclooxygense (Cox), also termed prostaglandin H synthase, is the enzyme catalyzing the rate-limiting step that converts free arachidonic acid to prostaglandin (PG) H_2 on the arachidonic cascade [1]. Presently, three isoforms, Cox-1, Cox-2, and Cox-3 have been identified. Cox-1 is present under normal conditions in most tissues and is responsible for housekeeping functions. On the other hand, Cox-2 is not normally present under the basal conditions or is present in very low amounts. However, it is rapidly induced in response to a wide variety of cytokines [2], growth factors [3], and ligands of G protein-coupled receptors [4]. The induction of the Cox-2 gene is regulated at both transcriptional (promoter-based) and post-transcriptional levels [3,5,6]. Intriguingly, Subbaramaiah et al. [7] have suggested that Cox-2 gene expression is negatively regulated by p53, implying functional interactions of Cox-2 with p53. Cox-3 is a splice variant of Cox-1 that shares the catalytic features of Cox-1 and Cox-2 and has a sensitivity for acetaminophen [8].

The tumor suppressor protein p53 is a transcription factor that regulates the response to a variety of stimuli such as DNA damage, hypoxia, oxidative stress, and oncogene expression [9].

Abbreviations: Cox, cyclooxygenase; EMSAs, electrophoretic movility shift assays; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HNE, 4-hydroxy-2-nonenal; MAPK, mitogen-activated protein kinase; RIPA, radioimmunoprecipitation assay; RT-PCR, reverse transcription-polymerase chain reaction; TTBS, tween 20/tris buffered saline

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http://dx.doi.org/10.1016/j.redox.2014.11.011

Inactivation of the p53 gene, either by mutation or deletion, has frequently been found in a variety of human malignant tumors [10,11]. Under normal conditions, p53 is a very labile protein. The rapid degradation of p53 is largely achieved through the ubiquitin–proteasome pathway. However, once cells are exposed to stimuli, the p53 protein increases promptly and regulates the various gene expressions. The accumulation of p53 protein in response to various stimuli occurs mainly through post-translational modification rather than the transcriptional level. p53 has many phosphorylation sites, and the phosphorylation status of p53 is thought to be involved in stabilization and function of the protein [12]. p53 exerts its role through the transcriptional regulation of genes involved in cell cycle control, DNA repair, senescence, and apoptosis. p53 increases the gene expression involved in the cell cycle and apoptosis such as p21 [13], MDM2 [14], Bcl-2 [15] and Bax [16]. On the other hand, p53 also represses the transcription of a number of genes, including topoisomerase IIα [17], MRP [18], and human reduced folate carrier [19].

Lipid peroxidation proceeds by a free radical chain reaction mechanism and yields lipid hydroperoxides as major initial reaction products. A key feature of the lipid peroxidation is the breakdown of these hydroperoxy fatty acids to yield a broad array of smaller fragments, 3–9 carbons in length, including reactive aldehydes, such as 2-alkenals and 4-hydroxy-2-alkenals [20–22]. There is increasing evidence that these aldehydes are causally involved in many of the pathophysiological effects associated with oxidative stress in cells and tissues. In view of the observation that liver injury associated with oxidative stress is accompanied by increased PG synthesis [23], it is hypothesized that lipid peroxidation products may be involved in the up-regulation of the PG biosynthesis. Our previous finding [24] that 4-hydroxy-2-nonenal (HNE), one of the major lipid peroxidation-derived electrophilic aldehydes, was identified as a potent Cox-2 inducer in rat epithelial RL34 cells supports this hypothesis. Moreover, we have revealed that HNE-induced Cox-2 expression resulted from the stabilization of Cox-2 mRNA that is mediated by the p38 mitogen-activated protein kinase (MAPK) signaling pathway [25]. In the present study, to examine whether the HNE-induced Cox-2 expression was mechanistically linked to the p53 expression, we analyzed changes in Cox-2 and p53 expression levels in response to HNE. Our data show that HNE-induced down-regulation of p53 is involved in the enhanced Cox-2 gene expression, probably through the activation of transcription factor Sp1. Moreover, using Cox-2-overexpressing derivatives of RL34 cells, we show that Cox-2 overproduction is associated with down-regulation of a proteasome subunit. The resultant proteasome dysfunction results in the enhanced accumulation of p53 and ubiquitinated proteins, leading to enhanced sensitivity toward electrophiles.

Experimental procedures

Materials

HNE was obtained from Cayman Chemical Co. (Ann Arbor, MI). Anti-Cox-2, anti-Sp1 and anti-p53 polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GenePORTER transfection reagent was obtained from Gene Therapy Systems, Inc. (San Diego, CA). and opti-MEM 1 reduced serum medium and the First-Strand cDNA synthesis kit were from Life Technologies, Inc. (Rockville, MD, USA). Horseradish peroxidase-linked anti-goat IgG was from DAKO Co. (A/S, Denmark). Anti-rabbit IgG immunoglobulin-conjugated horseradish peroxidase, enhanced chemiluminescence (ECL) western blotting detection reagents and Hybond ECL nitrocellulose membranes were obtained from Amersham (Piscataway, NJ). The protein concentration was measured using the BCA protein assay reagent obtained from Pierce Chemical Co.

Cell culture

RL34 rat liver epithelial-like cells were obtained from the Japanese Cancer Research Resources Bank. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing heat-inactivated 5% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (588 μg/ml), and 0.16% $NaHCO_3$. Cells were cultured in a 37 °C humidified atmosphere containing 95% air/5% CO_2. 50–60% Confluent cells were subjected to experiments in DMEM containing 5% FBS.

Cox-2 construct

Rat Cox-2 cDNA containing open reading frame was amplified by RT-PCR, using a set of primer that contained an EcoRV restriction site. After digestion with EcoRV, the amplified Cox-2 cDNA fragment was ligated into the EcoRV site of a linearized pCMV-Script expression vector (Stratagene, CA) using T4 DNA ligase to create the sense rat Cox-2 construct. The resulting expression plasmid was verified by restriction enzyme digestion and was named pcCox-2 vector. The primer used for rat Cox-2 full coding region amplification is as follows: (F), 5′-GATATCCTTCAGGAGTACGAAGACCCTGCCTAC-3′ and (R), 5′-GATATCATGGTAAGTAGACTCTTACAGCTCAG-3′.

Stable transfection with Cox-2 in RL34 cells

RL34 cells were transfected with pcCox-2 vector or with empty control vector using the GenePORTER™ transfection reagent (Gene Therapy Systems, Inc.). In these experiments, 1×10^6 cells were incubated with the DNA-GenePORTER mixture (2 μg of DNA/10 μl of GenePORTER) in 1 ml of serum-free Opti-MEM1 (GIBCO) at 37 °C. After 6 h of incubation, 1 ml of the complete medium was added, and the cells were cultured for 18 h. Thereafter, stable transfectants were isolated by culture in selection medium containing 700 μg/ml G418 for ~3 weeks. A single clone of the stably transfected cells was isolated and expanded. Several G418-resistant stable clones were maintained in regular growth medium containing 700 μg/ml G418.

PGD_2 assay

A solid phase enzyme immunoassay (Cayman Chemical) was performed as suggested by the manufacturer, and the PGD_2 level was determined using a standard curve and a linear log–logit transformation.

Oligonucleotide microarray

Total RNAs were isolated from the empty vector—the transfected control and Cox-2-transfected cells independently using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. The quality of the extracted total RNA was confirmed with an Agilent 2100 Bioanalyzer (Palo Alto, CA) or electrophoresis on 1.5% agarose gels stained by SYBR Green (Applied Biosystems, Foster City, CA). Double-stranded complementary DNA was synthesized from 10 μg of total RNA according to Affymetrix (Affymetrix, Santa Clara, CA) methodology, and cDNA was purified with Phase Lock Gels (Eppendorf, Hamburg, Germany). We synthesized biotin-labeled RNA with the BioArray High Yield RNA Transcript Labeling Kit (Enzo, New York, NY). Hybridization from biotinylated cRNA to murine genome GeneChips (MG-U74Av2, Affymetrix) was performed in accordance with the

manufacturer's instructions, stored at 40 °C overnight, and heated in a mix that included 10 μg fragmented RNA, 6 × SSPE, 0.005% Triton X-100, and 100 mg/ml herring sperm DNA in a total volume of 200 μl. The GeneChips were washed and stained with streptavidin-c (Molecular Probes, Eugene, OR), and probe arrays were scanned three times at 3-μm resolution using the GeneChip system confocal scanner made for Affymetrix by Hewlett-Packard. MicroArray Suite (MAS) version 5.1 (Affymetrix) was utilized to calculate from the scanned images. Intensity values were scaled such that the overall intensity for each GeneChip of the same type was equivalent [26]. The average difference of each experiment was normalized to 100 to allow comparison among multiple arrays.

Cell viability

Cell viability was quantified by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [27].

p53 antisense oligonucleotides transfection

Thiophosphorylated antisense oligonucleotides were purchased from Sigma Genosys Japan Co. RL34 cells were seeded at a density of 1×10^5 cells/35 mm dish and grown for 24 h in medium containing 5% FBS. Transient transfections were performed using the GenePORTER reagent according to the manufacturer's protocols. Briefly, the indicated concentration of oligonucleotides was prepared in 100 μl opti-MEM1 and incubated with 7 μl GenePORTER reagent prepared separately in 100 μl opti-MEM1. After 15 min, the DNA–reagent complexes were added to cells that had been washed with opti-MEM1 medium and incubated for 6 h. The medium was then changed to fresh medium containing 5% FBS, maintained for another 48 h, and subjected to the experiment.

Immunoblot analysis

The HNE-treated or untreated cells were washed twice with phosphate-buffered saline (pH 7.0) and lysed with RIPA buffer (50 mM Tris–HCl (pH 7.5)/150 mM NaCl/1% Triton X-100/0.5% sodium deoxycholate/0.1% SDS/100 μg/ml phenylmethylsulfonyl fluoride (PMSF)). Each whole cell lysate (total protein, 25 μg) was then treated with Laemmli sample buffer for 5 min at 100 °C [28]. The samples were run on 10% SDS-polyacrylamide gels and blotted on a nitrocellulose membrane (Amersham Bioscience), incubated with 4% Block Ace (Dainippon Pharmaceutical Co., Osaka, Japan) overnight at 4 °C for blocking, washed with TTBS, and treated with the antibodies to p53 or Sp1. This procedure was followed by the addition of horseradish peroxidase conjugated to IgG and ECL reagents. The bands were detected with Light-Capture (ATTO Co., Tokyo, Japan).

Immunoprecipitation

The whole cell lysate was prepared as described above, and 2 mg protein of each sample was incubated with protein A-sepharose beads (Pharmacia Bioscience) to remove nonspecific binding proteins at 4 °C for 1 h. After centrifugation, the supernatants were mixed with 2 μg of antibodies to p53 or Sp1 and then incubated at 4 °C with rotation overnight. Following the incubation with protein A-sepharose beads for 1 h, the antigen–antibody-beads complex was washed 4 times with the RIPA buffer and 3 times with PBS, boiled for 5 min after adding 30 μl of 3 × SDS-PAGE sample buffer and subjected to immunoblot analysis.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using ISOGEN reagent (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's protocol and spectrophotometrically quantified. First-strand cDNA was synthesized at 37 °C for 60 min with 5 μg of total RNA using an oligo(dT) primer and MMLV-RNA polymerase (Invitrogen). To stop the reaction the samples were heated at 95 °C for 2 min. PCR reactions were carried out in a final volume of 25 μl consisting of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.1% Triton-X 100, 200 μM amounts of each deoxyribonucleoside triphosphate, 0.5 μl of the RT first-strand cDNA product, 1 μM of each forward and reverse primer, and 2.5 units of r*Taq* DNA polymerase (Toyobo Co., Osaka, Japan). After the initial denaturation step for 3 min at 94 °C, 26 cycles of PCR were performed. Each cycle consisted of a denaturing step at 94 °C for 30 s, an annealing step at 54 °C for 30 s, and extension step at 72 °C for 30 s. After the cycling procedure, a final 10-min elongation step at 72 °C was performed. The following primers were used: Cox-2, (F) 5′-CCCTGCTGGTG GAAAAGCCTGGTCC-3′ and (R) 5′TACTGTAGGGTTAATG-TCATCTAG-3′; p53, (F) 5′-AGAGGAAGCCCTCCAAGTGTCA-3′ AND (R) 5′-ATAGT GGTATAGTCGGAG-3′. GAPDH, (F) 5′-AACCCATCACCATCTTCCAG-GAGC-3′ and (R) 5′-CACAGTCTTCTGAGTGGCAGTGAT-3′.

Nuclear extract preparation and electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared using RL34 cells following the standard protocol, with modifications. Briefly, cells were treated with reagents and washed with cold PBS, suspended in NP-40 buffer (10 mM HEPES (pH 7.6)/3 mM $MgCl_2$/40 mM KCl/2 mM DTT/5% glycerol/0.5% (v/v) NP-40/10 μg/ml aprotinin/10 μg/ml leupeptin/0.5 mM PMSF) and lysed by pipetting. Following incubation for 5 min on ice, the samples were centrifuged, and the supernatants were collected as the cytosol fraction. The pellets were washed with NP-40 buffer twice, resuspended in high salt buffer (10 mM HEPES (pH 7.9)/0.1 mM EGTA/420 mM NaCl/0.5 mM DTT/1.5 mM $MgCl_2$/0.5 mM PMSF/25% (v/v) glycerol), and incubated for 30 min at 4 °C with rotation. After centrifugation the supernatants were collected as the nuclear fraction. For EMSA, we used the 5′ GCAGAGGGCGGTGCAGCTCT 3′ oligonucleotide as a distal GC-rich element and 5′-AAAGC TGGGGGGGTGGGGGGGTGGGGAAAG-3′ oligonucleotide as a proximal GC-rich element. Nuclear extracts (10 μg protein) were preincubated with 2 μg of poly(dIdC) in 20 μl containing 20 mM HEPES-HCl (pH 7.9), 100 mM KCl, 4 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM PMSF, 10% glycerol for 15 min on ice before the addition of a ^{32}P-radiolabeled oligonucleotide probe (4×10^4 cpm). The incubation was continued for 20 min at room temperature. The samples were loaded onto a 4.5% polyacrylamide gel and electrophoresed at 20 mA in 0.5 × Tris-borate–EDTA buffer (450 mM Tris-borate (pH 8.0), and 1 mM EDTA) containing 2.5% glycerol. The gel was dried and exposed to an imaging plate and then analyzed by BAS2500 (Fuji Film Co, Japan). For supershift analysis, 2 μg of specific antibodies to Sp1 was added to the reaction mixture for 1 h on ice before the addition of a radiolabeled probe.

Immunofluorescence histochemistry

Cells were grown on coverstrips in a 24-well plate and then treated with the reagent at the indicated times. The cells were washed with cold PBS two times and fixed with 100% cold methanol for 20 min at −20 °C. The cells were then washed three times with PBS for 5 min, permeabilized with 0.5% Triton X-100 in PBS for 15 min, and washed three times with PBS (15 min each).

The coverstrips were incubated with 5% BSA in TTBS at 4 °C overnight for block nonspecific binding. Following washing with TTBS three times for 5 min, the coverstrips were incubated in TTBS containing anti-Sp1 antibodies for 1 h at room temperature. They were then washed with TTBS three times for 5 min and further incubated with TTBS containing anti-rabbit IgG-labeled FITC (Dako) for 1 h at room temperature. The coverstrips were then washed with TTBS three times for 10 min each and mounted. Images were all viewed using a confocal image microscope (BioRad).

Results

Inverse correlation between Cox-2 and p53 expression levels in response to HNE

To examine whether the HNE-induced Cox-2 expression was mechanistically linked to the p53 expression, changes in Cox-2 and p53 expression levels in response to HNE were analyzed. As shown in Fig. 1, HNE enhanced Cox-2 protein expression in a time-dependent manner. HNE induced a large and transient induction of Cox-2 that peaked at 3 h followed by a gradual decline to the control level after 24 h. Unlike Cox-2, Cox-1 expression levels remained unchanged in response to the HNE-induced stress (data not shown). In contrast, the p53 protein levels were significantly reduced during the first 3 h of the HNE treatment; however, the protein level of p53 thereafter began to recover and increased to over the basal level after 6 h of treatment. There was no apparent change in the p53 mRNA levels in response to the HNE treatment (data not shown). These data suggest the possibility that the Cox-2 protein level may be inversely correlated with the protein level of p53.

Proteasome-dependent down-regulation of p53 is involved in the Cox-2 expression

The hypothesis that p53 might be negatively correlated with Cox-2 expression was examined by the down-regulation of p53

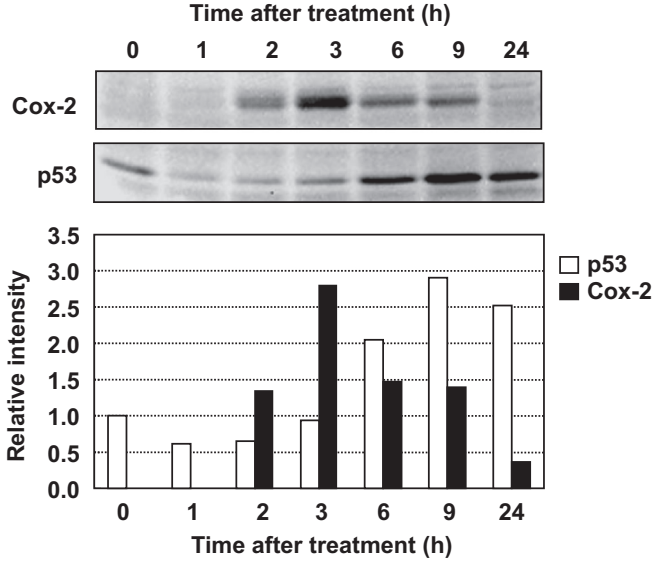

Fig. 1. Inverse correlation between Cox-2 and p53 expression levels in response to HNE. Induction of Cox-2 and p53 protein expressions in RL34 cells exposed to HNE. RL34 cells were treated with 25 μM HNE for different time intervals as indicated. Whole cell lysates (25 μg) were subjected to immunoblot analysis for detection of Cox-2 and p53. Relative intensities of Cox-2 and p53 proteins were estimated by comparing the intensity of basal level of p53.

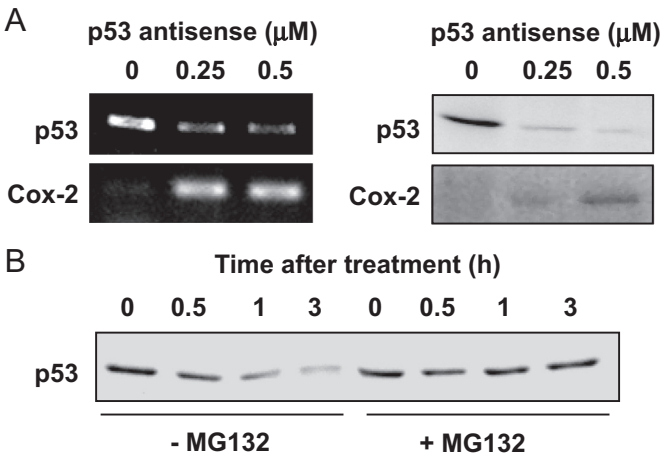

Fig. 2. Proteasome-dependent down-regulation of p53 is involved in the Cox-2 expression. (A) Effect of p53 antisense oligonucleotides on the expression of Cox-2 mRNA (left) and protein (right). Indicated doses of antisense oligonucleotides were transfected for 48 h in RL34 cells, and whole cell lysates and total RNA were then prepared for the analysis of p53 and Cox-2 protein levels and their mRNA levels, respectively. (B) Effect of a specific proteasome inhibitor on the HNE-induced down-regulation of p53. RL34 cells were pretreated with 1 μM MG132 and then treated with 25 μM HNE for the indicated times.

using antisense oligonucleotides against p53. the p53 antisense significantly enhanced the expression of Cox-2 mRNA and protein (Fig. 2A). The observation (Fig. 2B) that a specific proteasome inhibitor, MG132, significantly suppressed the HNE-induced down-regulation of p53 suggests that the proteasome should be responsible for the rapid reduction of p53 levels observed during the first 3 h of the HNE treatment. This is consistent with the fact that rapid demise of p53 is largely achieved through the ubiquitin/proteasome-dependent protein degradation pathway [12].

HNE stimulates dissociation of a p53–Sp1 heterocomplex

p53 regulates gene expression through binding to the consensus element within the promoter region. However, the rat Cox-2 promoter region has no putative p53-binding elements. This suggests the possibility that p53 directly binds to and acts on several cellular proteins, such as mdm2, SV40 T antigens, TATA binding proteins, and Sp1 that have been shown to interact with p53 [9,29]. In the present study, we focused on Sp1, because a minimal promoter region required for the basal transcription of the human Cox-2 gene contains GC-rich proximal sequences that are specifically bound by Sp1 [30]. Indeed, several lines of evidence have been reported to indicate molecular cross-talk between Sp1 and p53, including Sp1/p53-associated reciprocal [31], synergistic and cooperative regulation of target gene transcription [32,33]. To examine the functional interaction of p53 with Sp1, we first examined the formation of a p53–Sp1 heterocomplex in the cells exposed to HNE. As shown in Fig. 3A, the immunoprecipitation with an antibody to p53 or Sp1 followed by immunoblot analysis with the anti-Sp1 antibody revealed the presence of p53–Sp1 complexes in control and their dissociation by treatment with HNE. The HNE-induced dissociation of p53–Sp1 complexes was also confirmed by immunoprecipitation with an anti-Sp1 antibody followed by immunoblot analysis with the anti-p53 antibody (Fig. 3B). Moreover, HNE-induced dissociation of p53–Sp1 complexes was accompanied by nuclear translocation of Sp1 (Fig. 3C). These data indicate that p53 associates with Sp1 to an appreciable extent and that HNE enhances the dissociation of the p53–Sp1 complexes.

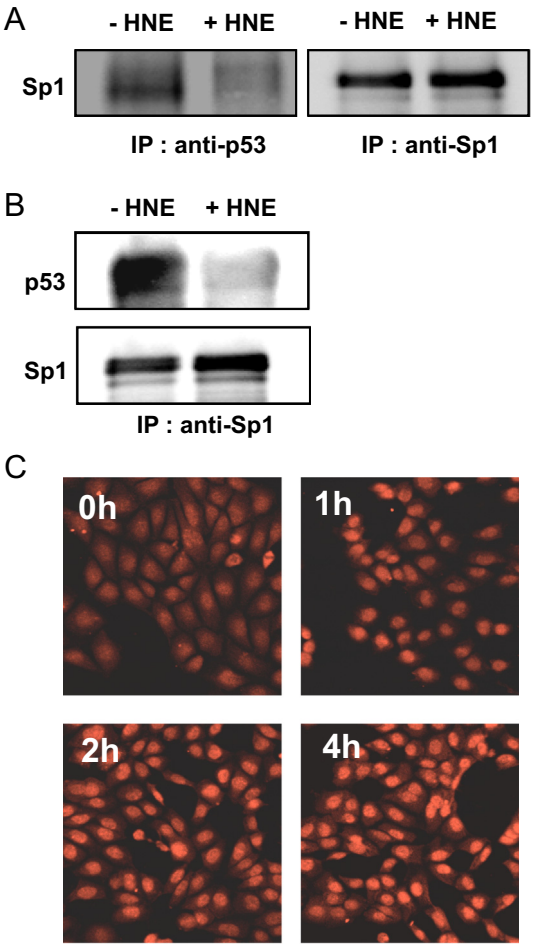

Fig. 3. HNE stimulates dissociation of a p53–Sp1 heterocomplex. (A) Immunoprecipitation with an antibody to p53 or Sp1 followed by immunoblot analysis with the anti-Sp1 antibody. (B) Immunoprecipitation with the anti-Sp1 antibody followed by immunoblot analysis with the anti-p53 antibody. In A and B, RL34 cells were treated with 25 μM HNE for 1 h. Immunoprecipitation followed by immunoblot analysis was performed as described in Experimental Procedures section. (C) Nuclear translocation of Sp1. RL34 cells were treated with 25 μM HNE for different time intervals as indicated. The cells were fixed with cold methanol and permeabilized with 0.5% Triton X-100/PBS and immunostained with the anti-Sp1 antibody. Images of the cellular immunofluorescence were acquired using a confocal laser scanning microscope.

Activation of Sp1

To examine whether HNE-induced dissociation of p53–Sp1 complexes and nuclear translocation of Sp1 were accompanied by activation of Sp1, we performed EMSAs using the oligonucleotide containing the Sp1 consensus element as a probe. Probes were described under Experimental Procedures section. As shown in Fig. 4A, two major DNA–protein complexes were observed. HNE treatment resulted in a time-dependent increase in the Sp1 DNA binding activity. Approximately a 2.5-fold increase in the Sp1–DNA binding activity was observed in the HNE-treated cells compared to the control. An almost maximal increase in Sp1 binding activity in response to HNE treatment was observed by 1 h, and these responses persisted for at least 3 h. The addition of antibody directed against Sp1 induced a supershift and a significant reduction in Sp1-dependent binding activity (Fig. 4B). Sp1 antibody mainly eliminated the upper complex. Although Sp1 abundance has been implicated in changes in Sp1 transcriptional activity [34], we did not see any changes in Sp1 level during the incubation periods (data not shown). Because Cox-2 gene expression is also transcriptionally regulated via the transactivation factor NF-κB p65 in human vascular endothelial cells [35], we investigated the involvement of NF-κB in the HNE-stimulated up-regulation of Cox-2. However, activation of NF-κB was not observed by EMSAs and by immunohistochemistry analyses (data not shown). Thus, Sp1 is likely to be involved in the HNE-stimulated gene expression of Cox-2. Taken together, down-regulation of p53 followed by the activation of transcription factor Sp1 was suggested to be involved in the HNE-induced Cox-2 gene expression.

Cox-2 overexpression causes down-regulation of a proteasome subunit

Although a causal role for Cox-2 has been proposed, mechanisms by which Cox-2 function contributes to the pathogenesis of hyperplastic disease are not well defined. As shown in Fig. 1, the recovery of p53 protein levels after 3 h of incubation seemed to be associated with the decrease in the protein level of Cox-2. To examine if there is any correlation between Cox-2 and p53 protein levels, we established the Cox-2-overexpressing derivatives of RL34 cells by stable transfection with Cox-2 cDNA. The Cox-2 expression vector (pcCox-2) was introduced into RL34 cells and four clones with resistance to G418 were selected. As a control, RL34 cells transfected with a control vector were similarly selected for G418 resistance. Eventually, three Cox-2-overexpressed cells (clone nos. 3, 8, 15) were isolated. As shown in Fig. 5, the Cox-2-transfected clone (no. 8) demonstrated a readily detectable expression of the Cox-2 protein by immunoblot analysis (*panel A*) and immunocytochemistry (*panel B*) with the anti-Cox-2 polyclonal antibody, whereas the control transfected cells did not. Stable transfection of Cox-2 into RL34 cells yielded comparable amounts of PGD_2 (*panel C*), suggesting that the Cox-2 polypeptide is enzymatically active.

To investigate the Cox-2-mediated change in gene expression, microarray analysis was used to examine the expression profile of a large number of transcripts. As shown in Table 1, we observed significant up-regulation of acetylcholinesterase-associated collagen, isopentenyl diphosphate–dimethylallyl diphosphate isomerase, and p38 MAPK genes. In addition, the expression of genes involved in the phase II detoxification response, such as glutathione S-transferase Yb and Yc subunits, was also significantly up-regulated. Meanwhile, we observed significant down-regulation of proteasome subunits RC1 and RN3, transforming growth factor beta-3, heat shock protein 27, apolipoprotein E, and prostacyclin synthase (Table 2). Most notably, the proteasome RC1 subunit was dramatically down-regulated by ~26-fold in the Cox-2 overexpressed cells. To confirm the microarray findings that the proteasome RC1 mRNA was down-regulated in Cox-2-overexpressed cells, RT-PCR was performed. As shown in Fig. 6, consistent with the microarray results, the levels of proteasome subunits RC1 and RN3, apolipoprotein E, and prostacyclin synthase were significantly down-regulated in the Cox-2-overexpressed cells. We determined whether prostanoid secretion by the Cox-2-transfected cells is necessary for the down-regulation of proteasome RC1 subunit caused by Cox-2. However, a series of Cox inhibitors, such as dexamethasone, nimesulide, ibuprofen, and NS398, were inactive in the recovery of the proteasome RC1 subunit in the Cox-2-overexpressed cells (Supplementary data, Fig. S1), suggesting the prostanoid-independent down-regulation of the proteasome subunit.

Enhanced accumulation of p53 and ubiquitinated proteins in the Cox-2-overexpressed cells

Accompanied by the down-regulation of the proteasome subunit, the proteasome activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase activities) were

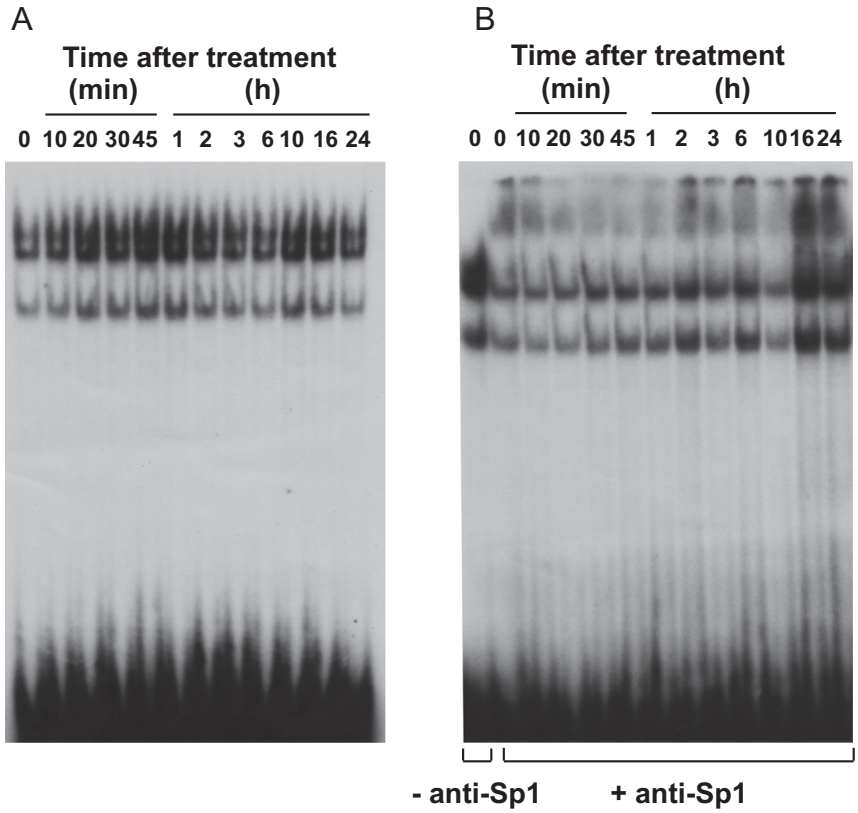

Fig. 4. Activation of Sp1 by HNE. EMSAs were performed using the oligonucleotide containing the Sp1 consensus element as a probe. (A) Time-dependent activation of Sp1. Nuclear extracts from RL34 cells treated or untreated with 25 μM HNE for the indicated times were incubated with the ^{32}P-labeled Sp1 oligonucleotides and separated by 4.5% acrylamide gel electrophoresis. (B) Nuclear extracts were prepared from RL34 cells treated with 25 μM HNE for the indicated times, preincubated with or without Sp1 antibody prior to the addition of the radiolabeled probe and separated by 4.5% acrylamide gel electrophoresis.

moderately down-regulated in the Cox-2-overexpressed cells (Fig. 7A). Because proteasome is responsible for the majority of cellular proteolysis in eukaryotic cells and contributes to controlling the intracellular levels of a variety of short-lived proteins [36–38], it was anticipated that this Cox-2-mediated dysfunction of proteasome might influence the turnover of proteasome substrates. Substrates of proteasome include a number of cell regulatory molecules, such as p53. Indeed, we observed that the basal level of p53 in Cox-2-overexpressed cells was significantly higher than that in the empty-vector transfected control cells (Fig. 7B). No significant morphological and cell proliferative difference in COX-2 overexpressing cells compared to control cells were observed. The functional impact of Cox-2 overexpression on proteasome activity was also evaluated by the accumulation of ubiquitinated proteins in response to HNE. As shown in Fig. 7C, a steady-state level of ubiquitinated proteins was found to be higher in the Cox-2-overexpressed cells than in the control cells. Moreover, the HNE-induced accumulation of ubiquitinated proteins was significantly enhanced by the Cox-2 overexpression.

Sensitivity of Cox-2-overexpressed cells to electrophiles

Finally, we examined the effect of Cox-2 overexpression on the cytotoxicity of electrophiles. One control and three Cox-2-transfected cells (clone nos. 3, 8, and 15) were examined by MTT assay for sensitivity to the cytotoxicity induced by exposure to HNE, showing that HNE (50 μM) resulted in a decrease in the MTT reduction levels to 63% of the basal levels in vector control cells and to 10% of the basal levels in Cox-2-overexpressed cells after 9 h (Fig. 8, panels A and B). Cytotoxicity induced by other electrophiles, tert-butylhydroquinone and diethylmaleate, was also enhanced in the Cox-2-overexpressed cells compared with the control cells (Fig. 8C). These results suggest that the proteasome dysfunction mediated by the Cox-2 overproduction influences cellular integrity and makes the cells sensitive to electrophiles.

Discussion

Under oxidative stress, HNE is believed to be produced in relatively high amounts *in vivo*, reaching concentrations of 10 μM to 5 mM in response to oxidative insults [21]. Moreover, HNE, which has an electrophilic property, attacks many molecules such as proteins and nucleotides and forms adducts with them, thus causing dysfunction of the target molecules. In particular, the reaction between HNE and proteins has been well established. HNE has the ability to form adducts with proteins by interacting with either the sulfhydryl groups of cysteine or the amino groups of lysine and histidine [39]. By immunohistochemical analysis, HNE–protein adducts have been detected in types of disorder foci from liver disease to neuronal disease [39]. Interestingly, Cox-2 expression or prostaglandin overproduction is also seen in several of the same disease foci, such as atherosclerosis and Alzheimer's disease [40,41]. In the liver, it is known that oxidative stress is increased with alcohol feeding and is accompanied by lipid peroxidation products such as HNE [42]. Moreover, in an alcohol-fed rat, which is a model of alcoholic liver disease, alcohol over-intake increases HNE-modified protein formation and is associated with the Cox-2 and proinflammatory cytokine TNF-α expression [23,43]. These findings suggest the possibility that lipid peroxidation products may be involved in the up-regulation of the prostaglandin biosynthesis.

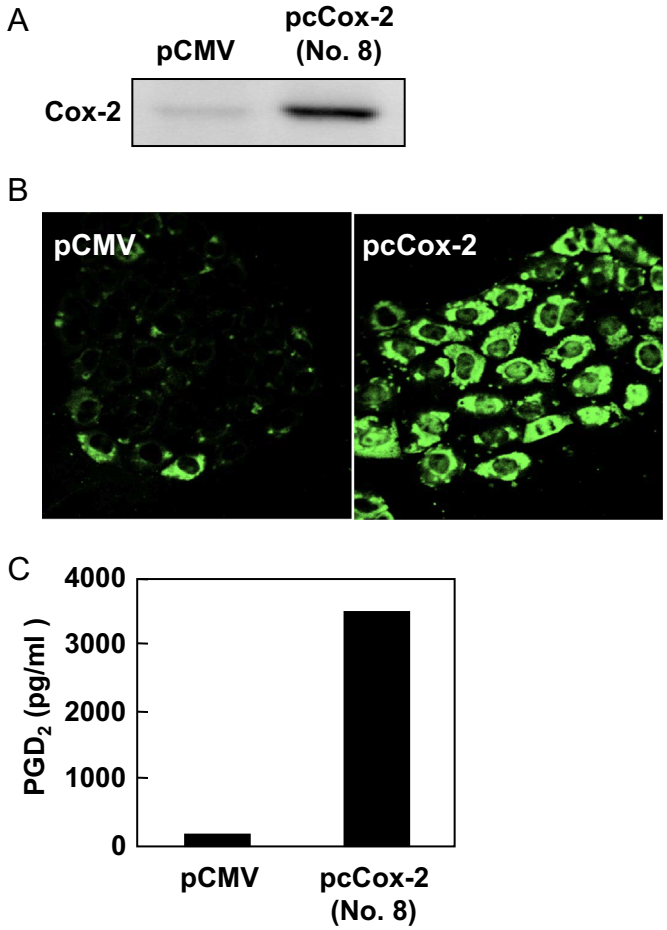

Fig. 5. Preparation of Cox-2-overexpressing derivatives of RL34 cells. The Cox-2 expression vector (pcCox-2) was introduced into RL34 cells, and three clones (clone nos. 3, 8, 15) with resistance to G418 were selected. As a control, RL34 cells transfected with a control vector and were similarly selected for G418 resistance. The levels of Cox-2 protein in the control transfected cells and the Cox-2-overexpressed cells (clone no. 8) were examined by immunoblot analysis (A) and immunocytochemistry (B) with the anti-Cox-2 monoclonal antibody. (C) The levels of PGD_2 in the control transfected cells and the Cox-2-overexpressed cells (clone no. 8).

Accumulating evidence indicates that Cox-2 activity and prostaglandin synthesis play an important role in the promotion of various proliferative diseases including cancer. It is well documented that Cox-2 can be induced by growth factors and cytokines, inflammatory stimuli and tumor promoters [44–47]. Recent studies have provided evidence that oxidative stresses as well as DNA-damaging agents, including ionizing radiation, can induce Cox-2 expression [48–50]. However, the mechanism of induction of Cox-2 in response to oxidative stress or DNA damage remains unknown. In our previous study, based on the experimental evidence [23] that oxidative stress is closely associated with the up-regulation of Cox-2, we evaluated the effect of the oxidized fatty acid metabolites on Cox-2 induction in rat liver epithelial RL34 cells and found that, among the oxidized fatty acid metabolites tested, only HNE showed the inducibility of Cox-2 expression [24]. In addition, we investigated the molecular mechanism underlying the Cox-2 induction by HNE and established that the p38 mitogen-activated protein kinase pathway, which exerts its function by increasing the stability of Cox-2 mRNA, plays a key role in the mechanism of HNE-induced Cox-2 expression in RL34 cells [25]. In the present study, to investigate transcriptional regulation of the Cox-2 gene in response to HNE, we examined whether the HNE-induced Cox-2 was mechanistically linked to the p53 expression and found that the Cox-2 levels were inversely correlated with the p53 levels (Fig. 1). In addition, the down-regulation of p53 with the antisense oligonucleotides against p53 significantly enhanced the expression of Cox-2 mRNA and protein (Fig. 2A). These observations are important for several reasons. For example, suppression of Cox-2 expression by p53 can explain why levels of Cox-2 protein are undetectable in normal epithelial cells and, by contrast, why mutations of p53 may contribute to the increased expression of Cox-2 that is observed in malignant tissues [51–55].

p53 regulates gene expression through binding to the consensus element within the promoter region. However, it is also known that p53 suppresses a variety of promoters that contain TATA elements [56–58]. This suppression is thought to occur through direct interaction with components of the basal transcription machinery, such as TAF70, TFIIH, and TATA binding proteins. On the other hand, based on the following observations, we hypothesized that Sp1, a general transcription factor that is involved in various inducible and constitutive gene expressions, is also involved in the induction of Cox-2 in response to HNE. (*i*) p53 suppresses various gene expressions through preventing Sp1 activity [59], (*ii*) The rat Cox-2 promoter region has no putative p53-binding elements, and a minimal promoter region required for the basal transcription of the human Cox-2 gene has been demonstrated to contain GC-rich proximal sequences that are specifically bound by Sp1 [60], and (*iii*) p53 negatively regulates Sp1 through the formation of a p53–Sp1 heterocomplex [61,62]. Indeed, the immunoprecipitation experiments indicated that p53 bound to Sp1 in intact cells under normal conditions and that HNE stimuli elicited the dissociation (Fig. 3, panels A and B). It was also observed that the dissociation of p53–Sp1 complexes was accompanied by the nuclear translocation of Sp1 (Fig. 3C). To examine whether HNE-induced dissociation of p53–Sp1 complexes and nuclear translocation of Sp1 were accompanied by activation of Sp1, we performed EMSAs using the oligonucleotide containing the Sp1 consensus element as a probe and observed that HNE treatment resulted in a time-dependent increase in the Sp1 DNA binding activity (Fig. 4). Although the regulatory mechanism of dissociation of p53–Sp1 complex remains unclear, the involvement of a phosphatidylinositol 3-kinase pathway, which induces p53 degradation through mdm2 phosphorylation [63–65], may not be unlikely. This possibility is supported by our finding that HNE activates phosphatidylinositol 3-kinase/AKT pathway in vascular smooth muscle cells [66] and our observation that wortmannin, a specific inhibitor of phosphatidylinositol 3-kinase, significantly inhibited the Cox-2 expression (T. Kumagai and K. Uchida, unpublished data). Taken together, it is hypothesized that down-regulation of p53 followed by the activation of a transcription factor Sp1 may be involved in the HNE-induced Cox-2 gene expression (Fig. 9).

Aberrant up-regulation of Cox-2 expression is increasingly implicated in the pathogenesis of epithelial cell carcinomas such as human colorectal adenocarcinomas and colorectal tumors [67]. Previous studies in rat medullary interstitial cells have shown that Cox-2 overexpression leads to a number of effects that could be associated with tumorigenesis: increased adhesion to extracellular matrix proteins, inhibition of butyrate-induced apoptosis, decreased expression of both E-cadherin and transforming growth factor 2 receptor, and stimulation of Bcl-2 protein expression [68]. Cox-2-expressing cells also produce high levels of angiogenic factors that stimulate angiogenesis [69]. It has also been shown that the sustained presence of Cox-2 is involved in the regulation of cell cycle arrest [70]. Indeed, treatment of vascular endothelial cells with the cytokine interleukin-1 or tumor promoter PMA results in a concomitant increase in Cox-2 expression and inhibition of cell growth [71].

Table 1
Cox-2-inducible genes in rat liver epithelial RL34 cells.

Accession no.	Gene name	Fold
AF007583	Rattus norvegicus acetylcholinesterase-associated collagen (COLQ) mRNA, complete cds	2.04
AF003835	Rattus norvegicus isopentenyl diphosphate–dimethylallyl diphosphate isomerase mRNA, complete cds	1.98
RNU73142	Rattus norvegicus p38 mitogen activated protein kinase mRNA, complete cds	1.84
AF025506	Rattus norvegicus prenylated rab acceptor 1 (PRA1) mRNA, complete cds	1.78
AF076183	Rattus norvegicus cytosolic sorting protein PACS-1a (PACS-1) mRNA, complete cds	1.69
RATSIRR2	RATSIRR2 Rat mRNA for sIRR-2 (insulin receptor-related receptor alternatively spliced product), complete cds	1.67
RNCF6M	Rattus norvegicus mRNA for coupling factor 6 of mitochondrial ATP synthase complex	1.67
AF062594	Rattus norvegicus nucleosome assembly protein mRNA, complete cds	1.66
RATRBP6A	Rat insulin-like growth factor binding protein (rIGFBP-6) mRNA, complete cds	1.62
AB011679	Rattus norvegicus mRNA for class I beta-tubulin, complete cds	1.60
RATFN3M1	L00191cds#1 RATFN3M1 Rat fibronectin (cell-, heparin-, and fibrin-binding domains) gene encoding three fibronectin mRNAs, exons 1, 2, 3	1.57
RATIP3R3X	Rat inositol trisphosphate receptor subtype 3 (IP3R-3) mRNA, complete cds	1.55
RATGSTYC	Rat liver glutathione S-transferase Yc subunit mRNA, complete cds	1.52
AB005549	Rattus norvegicus mRNA for atypical PKC specific binding protein, complete cds	1.51
RATCSBP	D17711cds RATCSBP Rat mRNA for dC-stretch binding protein (CSBP), complete cds	1.49
RNU95178	Rattus norvegicus DOC-2p59 isoform mRNA, complete cds	1.49
RATPRORR12	RATPRORR12 Rat mRNA for proteasome subunit R-RING12, complete cds	1.49
RNGSTYBR	X04229cds RNGSTYBR Rat mRNA for glutathione S-transferase (GST) Y(b) subunit (EC 2.5.1.18)	1.45
AB011532	Rattus norvegicus mRNA for MEGF6, complete cds	1.44
RATBTG1H	Rattus norvegicus anti-proliferative factor (BTG1) mRNA, complete cds	1.44
AF031878	Rattus norvegicus peripherin mRNA, complete cds	1.44
RATTYRPHOS	RATTYRPHOS Rat protein tyrosine phosphatase mRNA, complete cds	1.43
RATESP1A	RATESP1A Rattus rattus R-esp1 mRNA, complete cds	1.42
RATRIP	M24542cds RATRIP Rat Rieske iron–sulfur protein mRNA, complete cds	1.42
RRRPL21	X15216 cds RRRPL21 Rattus rattus mRNA for ribosomal protein L21	1.41

The top 25 genes that are up-regulated in the Cox-2-overexpressed cells are shown in the order of the values of the ratio of expression levels between control and Cox-2-overexpressed cells.

Table 2
Cox-2-repressed genes in rat liver epithelial RL34 cells.

Accession no.	Gene name	Fold
RATPSRC1	RATPSRC1 Rat mRNA for proteasome subunit RC1	0.04
RRU03491	Rattus norvegicus Wistar transforming growth factor beta-3 mRNA, complete cds	0.28
RATHSP27A	M86389cds RATHSP27A Rat heat shock protein (Hsp27) mRNA, complete cds	0.36
RATMEP	Rat metalloendopeptidase mRNA, complete cds	0.42
RNU53855	Rattus norvegicus prostacyclin synthase (ratpgis) mRNA, complete cds	0.44
RRU07619	Rattus norvegicus Sprague-Dawley tissue factor protein mRNA, complete cds	0.45
AF045564	Rattus norvegicus development-related protein mRNA, complete cds	0.45
AB014722	Rattus norvegicus mRNA for rSALT-1(806), complete cds	0.46
AF016047	Rattus norvegicus platelet-activating factor acetylhydrolase alpha 1 subunit (PAF-AH alpha 1) gene, complete cds	0.47
RNU34932	Rattus norvegicus Fos-related antigen mRNA, complete cds	0.47
AF057564	Rattus norvegicus putative retrovirus-related gag protein mRNA, complete cds	0.48
AF051155	Rattus norvegicus G beta-like protein GBL mRNA, complete cds	0.48
RNU18729	Rattus norvegicus cytochrome b558 alpha-subunit mRNA, complete cds	0.51
AB003991	rat mRNA for SNAP-25A, complete cds.	0.51
S76054	cytokeratin-8 [rats, prostatic epithelia, mRNA, 1747 nt]	0.53
RATSCPIIX	Rat voltage-dependent sodium channel type II protein gene, complete cds.	0.60
RNU19893	Rattus norvegicus alpha actinin mRNA, complete cds	0.61
RNAPOEG	Rat gene for apolipoprotein E.	0.61
RNEP2GL	X56327cds RNEP2GL Rattus norvegicus epsilon 2 globin gene	0.63
RATBCCAPA	Rattus norvegicus beta'-chain clathrin associated protein complex AP-1 mRNA, complete cds	0.64
RATCYP2A1	M33312cds RATCYP2A1 Rat hepatic steroid hydroxylase IIA1 (CYP2A1) gene, complete cds	0.64
D89514	Rattus norvegicus mRNA for 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase, complete cds	0.64
AF104399	Rattus norvegicus melanocyte-specific gene 1 protein (msg1) mRNA, complete cds	0.65
S75280	Rattus sp. pre-mtHSP70 mRNA complete cds; nuclear gene for mitochondrial product	0.65
RATTRO2A	Rat alpha-tropomyosin 2 mRNA, complete cds	0.66
RNGPXIMR	X12367 cds RNGPXIMR Rattus norvegicus mRNA for glutathione peroxidase I	0.66
RRLENSABC	X60351 cds RRLENSABC Rattus rattus mRNA for alpha B-crystallin (ocular lens tissue)	0.66
RATSERINE	Rattus norvegicus serine protease gene, complete cds	0.67
RATCTTG	Rat troponin T cardiac isoform gene, complete cds	0.67
RNU16253	Rattus norvegicus corticotropin-releasing factor receptor subtype 2 (CRF2R) mRNA, complete cds.	0.67
S78556	grp75=75 kDa glucose regulated protein [rats, Sprague-Dawley, brain, mRNA, 3001 nt]	0.68
RATCRYAB	Rat alpha-crystallin B chain mRNA, complete cds	0.68
RNU31880	Rattus norvegicus eIF-2B beta subunit mRNA, complete cds	0.69
AF074609	AF074609mRNA Rattus norvegicus MHC class I antigen (RT1.EC3) gene, complete cds	0.70
RATRN3	RATRN3 Rattus norvegicus proteasome RN3 subunit mRNA, complete cds	0.70

The top 35 genes that are down-regulated in the Cox-2-overexpressed cells are shown in the order of the values of the ratio of expression levels between control and Cox-2-overexpressed cells.

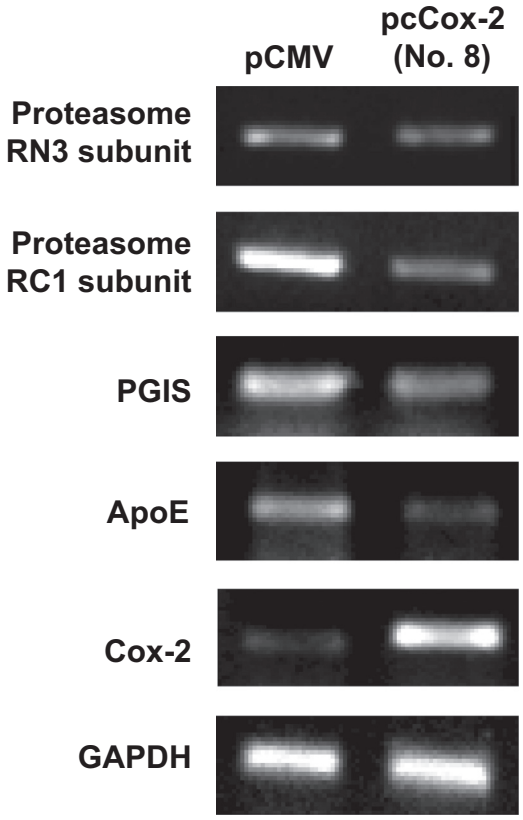

Fig. 6. Cox-2 overexpression causes down-regulation of a proteasome subunit. Gene expression of proteasome subunits RC1 and RN3, apolipoprotein E, and prostacyclin synthase in the control transfected cells and the Cox-2-overexpressed cells (clone no. 8) was analyzed by RT-PCR.

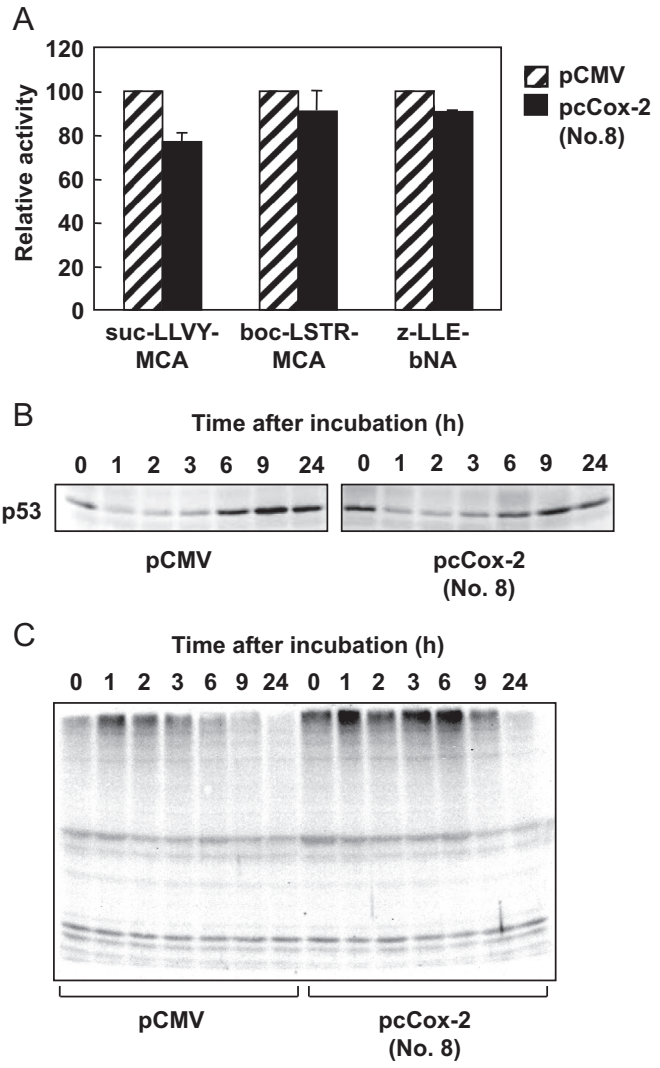

Fig. 7. Enhanced accumulation of p53 and ubiquitinated proteins in the Cox-2-overexpressed cells. (A) Effect of Cox-2 overexpression on proteasome activity in RL34 cells. *Bars*: *hatched bars*, control transfected cells; *closed bars*, Cox-2-overexpressed cells. The results shown are the mean ± S.E.M. of 3 independent experiments. The proteasome activities were measured using the fluoropeptides, s-LLVY-MCA, for the chymotrypsin-like activity, Boc-LSTR-MCA for the trypsin-like activity, and Z-LLE-βNA for the peptidylglutamyl peptide hydrolase activity, as proteolytic substrates. The results shown are the mean ± S.E.M. of 3 independent experiments. (B) HNE-induced accumulation of p53 in the control transfected cells and the Cox-2-overexpressed cells (clone no. 8). (C) HNE-induced accumulation of ubiquitin–protein conjugates in the control transfected cells and the Cox-2-overexpressed cells (clone no. 8). In panels B and C, both cells were incubated with 25 μM HNE at 37 °C.

The present study utilized gene expression profiling to assess thousands of genes to obtain a more detailed understanding of the molecular programs utilized by Cox-2-overexpressed cells. One important outcome from this study includes the identification of a link between sustained overexpression of Cox-2 and proteasome dysfunction. Proteasome is a large multisubunit protease complex that selectively degrades intracellular proteins [72–74]. Most of the proteins removed by these proteases are tagged for destruction by ubiquitination. Proteasome has a role to play in controlling cellular processes, such as metabolism and the cell cycle, through signal-mediated proteolysis of key enzymes and regulatory proteins. It also operates in the stress response by removing abnormal proteins and in the immune response by generating antigenic peptides. A decreased capacity for protein degradation is related to several degenerative diseases, such as Parkinson's disease, Alzheimer's disease, and amyotrophic lateral sclerosis, in which accumulation of abnormal polypeptides within cells leads to the death of neurons, as well as diabetes and atherosclerosis. An altered ubiquitin–proteasome system and reduced proteasome activity are associated with some of these diseases [75–78]. In the present study, Cox-2 overexpression in RL34 cells resulted in specific down-regulation of the proteasome subunit RC1 (Table 2 and Fig. 6). The proteasome dysfunction mediated by the Cox-2 overproduction was associated with (*i*) a moderate reduction of the proteasome activities (chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolase activities) (Fig. 7A), (*ii*) the accumulation of detrimental proteins, such as p53 (B) and ubiquitinated proteins (C), and (*iii*) enhanced sensitivity toward electrophiles (Fig. 8). The observation that the protein level of p53 began to increase after the Cox-2 level reached a maximum at 3 h (Fig. 1A) suggests that the accumulation of p53 may be associated with the down-regulation of Cox-2 through binding to Sp1 (Fig. 10). Alternatively, based on our previous observation that 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, a metabolite of PGD_2, inactivates proteasome through covalent modification [79], the decrease in the proteasome activity observed in this study may also be caused by the PG metabolites.

It has been shown that the overall structure of the proteasome RCl is almost identical to that of Ring10, whose gene is located in the class II region of the human MHC gene cluster [80]. In the meantime, it also appeared that the ability of Cox-2 to induce down-regulation of the proteasome subunit RC1 was independent of prostanoid secretion (Supplementary data, Fig. S1). This prostanoid-independent effect of Cox-2 is not unique to the

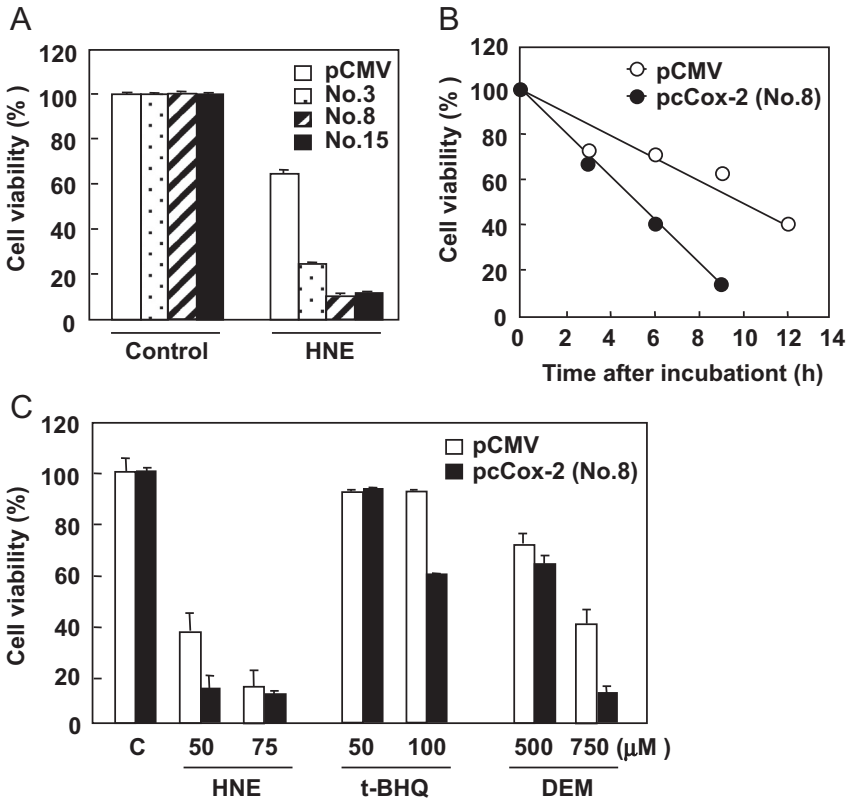

Fig. 8. Sensitivity of Cox-2-overexpressed cells to electrophiles. (A) HNE cytotoxicity to the control and Cox-2-overexpressed cells. One control and three Cox-2-transfected cells (clone nos. 3, 8, and 15) were examined by MTT assay for sensitivity to HNE (50 μM). (B) Time-dependent reduction of cell viability induced by HNE (50 μM) in the control transfected cells and the Cox-2-overexpressed cells (clone no. 8). (C) Electrophile cytotoxicity to the control and Cox-2-overexpressed cells. The control transfected cells and the Cox-2-overexpressed cells (clone no. 8) were examined by MTT assay for sensitivity to HNE (50 or 75 μM), butylhydroquinone (50 or 100 μM), and diethylmaleimide (500 or 750 μM) for 24 h.

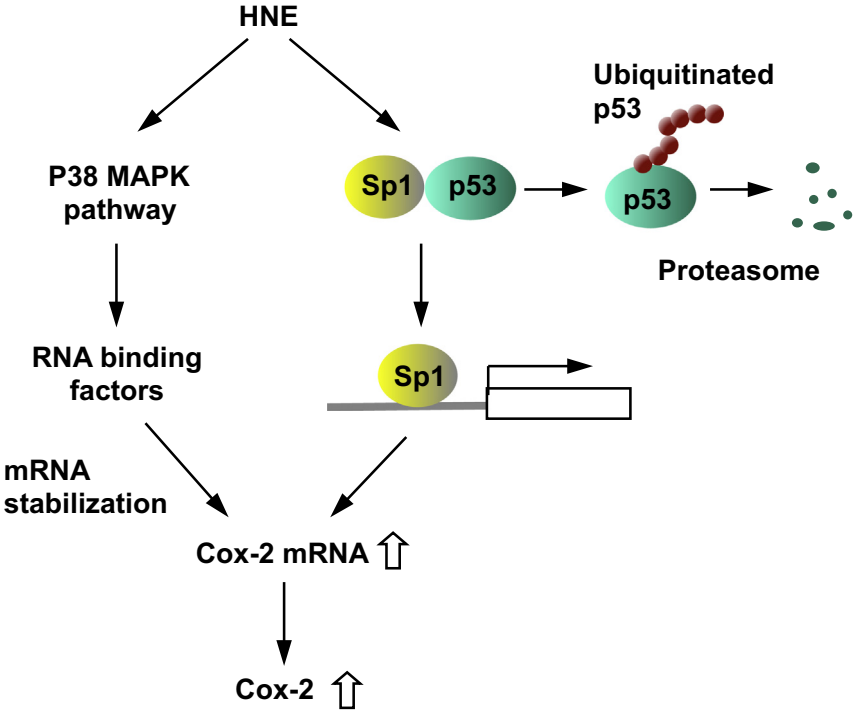

Fig. 9. Model for mechanisms by which HNE up-regulates Cox-2. HNE stabilizes Cox-2 mRNA through the p38 MAPK signaling pathway, leading to the up-regulation of Cox-2 [24]. On the other hand, the present work suggests an alternative mechanism, by which HNE induces Cox-2 gene expression through down-regulation of p53 followed by the activation of Sp1.

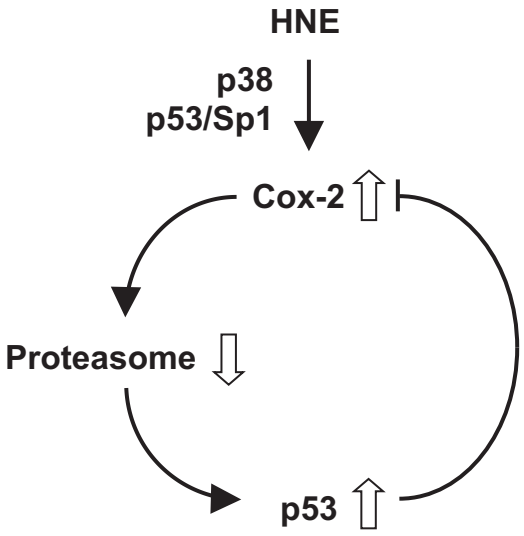

Fig. 10. Regulatory mechanism of Cox-2 gene expression mediated by proteasome dysfunction followed by p53 up-regulation.

proteasome subunit. Indeed, it has previously been shown that interleukin-1 and PMA-induced endothelial cell growth arrest is not reversed by nonsteroidal anti-inflammatory drugs that block prostanoid synthesis [71] and that neither NS-398 (a Cox-2-specific inhibitor) nor indomethacin could reverse the effect of Cox-2 overexpression on cell cycle progression [70]. Although the detailed mechanisms by which Cox-2 overexpression down-regulated the proteasome subunit remain unclear, interaction of the Cox-2 polypeptide with regulatory protein(s) on gene expression of the proteasome subunit may not be unlikely. Indeed, Cox-2 was shown to bind to an apoptosis regulatory protein nucleobindin [81]. Nevertheless, data in this report show unequivocally that Cox-2 overexpression induces proteasome dysfunction by an uncharacterized nonprostanoid-dependent signaling pathway. Further studies are required to define at a molecular level this novel mechanism of Cox-2 function and to assess its physiological relevance.

Acknowledgments

We thank Ms. Yuki Hondoh for her excellent editorial support. This work was supported in part by COST CM 1001; Grants-in-Aid for Scientific Research (numbers: 21248016 and 24658122), Grants-in-Aid for Scientific Research on Innovative Areas (Research in a Proposed Area) (number: 20117007), JST A-step program (number: AS242Z01310Q), and JST PRESTO program (number: 10604) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT).

Appendix A. Supplementary data

The supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2014.11.011.

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